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REVIEW

Friendly fire: redirecting herpes simplex virus-1 for therapeutic applications

S. J. Advani[1,2], R. R. Weichselbaum[2], R. J. Whitley[3] and B. Roizman[1]

[1]The Marjorie B. Kovler Viral Oncology Laboratories, [2]The Department of Radiation and Cellular Oncology, The University of Chicago, Chicago, Illinois and [3]The Departments of Pediatrics, Medicine, and Microbiology, The University of Alabama at Birmingham, Birmingham, Alabama, USA

Herpes simplex virus-1 (HSV-1) is a relatively large double-stranded DNA virus encoding at least 89 proteins with well characterized disease pathology. An understanding of the functions of viral proteins together with the ability to genetically engineer specific viral mutants has led to the development of attenuated HSV-1 for gene therapy. This review highlights the progress in creating attenuated genetically engineered HSV-1 mutants that are either replication competent (viral non-essential gene deleted) or replication defective (viral essential gene deleted). The choice between a replication-competent or -defective virus is based on the end-goal of the therapeutic intervention. Replication-competent HSV-1 mutants have primarily been employed as antitumor oncolytic viruses, with the lytic nature of the virus harnessed to destroy tumor cells selectively. In replacement gene therapy, replication-defective viruses have been utilized as delivery vectors. The advantages of HSV-1 vectors are that they infect quiescent and dividing cells efficiently and can encode for relatively large transgenes.

Keywords Gene therapy, HSV-1, oncolysis

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Gene therapy utilizing genetically engineered herpes simplex virus-1 (HSV-1) is under active investigation for the treatment of both cancer and somatic diseases. Wild-type HSV-1, however, is a highly pathogenic virus. Following replication at the portal of entry (mucosal epithelial tissue) with subsequent lysis of the infected cell, the virus infects sensory neurons (neuroinvasion) and is transported retrogradely to the nucleus of the neuron. Here the virus enters either a lytic or latent state. The lytic cycle (neurovirulence) is characterized by productive viral infection with the release of progeny virions, and, in the worst case, causes fatal encephalitis. Establishing latency in sensory ganglia is a hallmark of HSV infection; however, this process is poorly understood. In the latent state, the viral DNA remains extrachromosomal; the only transcribed RNA appears to be that of the latency-associated transcripts (LATs). These biological properties of wild-type HSV-1 have also made it a focus of intense study for gene therapy applications. Its ability to destroy cells in the lytic cycle has been exploited for cancer therapy with attenuated replication-competent HSV-1. Its tropism for neuronal cells (i.e. postmitotic cells) has been harnessed for treating neurodegenerative diseases, and especially intriguing in this treatment paradigm is its ability to peacefully coexist within the neuron in a latent state and transcribe the *LAT* gene. Theoretically, the large transgene coding capacity of HSV-1 also offers advantages over other viruses, as it can be a vector for the delivery of relatively large foreign genes.

HSV-1 is an enveloped, double-stranded DNA virus with a viral genome of 152 kb encoding at least 84 polypeptides [1]. The genome has two unique stretches, the unique long (U_L) and unique short (U_S) segments, separated by terminal inverted repeats. Genes have traditionally been classified as essential or non-essential, depending upon their requirement for viral replication in cell culture. However, even the non-essential genes play an important role in HSV-1 replication within

Corresponding author and reprint requests: S. J. Advani, Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street, Chicago, IL 60637, USA
Tel: +1 773 702 1898
Fax: +1 773 702 3791
E-mail: sjadvani@rover.uchicago.edu

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its natural host. Viral gene expression occurs in a temporal cascade, with immediate early (α) genes expressed first, followed by early (β) and then late (γ) gene expression. The products of α genes are infected cell proteins 0 (ICP0), ICP4, ICP22, ICP27, and ICP47, and are primarily involved in transcriptional regulation. β gene products are involved in viral DNA replication, and γ gene products primarily encode structural proteins.

The use of genetically engineered HSV-1 for gene therapy involves either replication-competent or replication-defective HSV-1 mutants. Replication-competent viruses have deletions in non-essential genes that attenuate the virus such that replication is dependent on the type of cell that the virus infects. For example, replication-competent HSV-1 mutants for tumor therapy selectively replicate more efficiently in tumor cells than in normal cells. Replication-defective HSV-1 mutants are deleted in one or more essential genes and therefore are unable to enter the replication program. The use of replication-competent or -defective HSV-1 mutants for gene therapy applications depends on the goal of therapy. For cancer therapy, replication-competent HSV-1 mutants have provided a powerful tool for tumor cell destruction. In the therapy of neurodegenerative diseases, the construction of replication-defective HSV-1 mutants that encode and deliver protective genes without cell destruction has been studied in experimental models.

ATTENUATED REPLICATION-COMPETENT HSV-1 FOR CANCER THERAPY

The concept of viral lysis of tumor cells has been discussed for more than a century [2]. Viral oncolysis is based on a simple yet elegant approach to cancer treatment. A principle consequence of the replicative cycle of selected viruses is cell lysis with subsequent spread to and lysis of adjacent tumor cells. In theory, infection of a single cancer cell within a tumor initiates a self-sustaining chain reaction resulting in infection and cell death throughout the tumor. The key to such an approach is genetically engineering viral attenuation such that the virus is predisposed to replicate in cancer cells but not the adjacent normal tissue. Replication-competent, attenuated HSV and adenovirus as well as reovirus are currently being employed as such antitumor agents [3–5].

The central tenet of this approach is the ability to create a virus that targets tumor cells for death and not the non-transformed adjacent cells. Theoretically, this problem can be addressed at multiple points during the virus life cycle, beginning with the attachment of the virus to the target cell. Upon entering the cell, the lytic program of the virus can be controlled by the intracellular environment such that it replicates within rapidly dividing cells (i.e. tumor cells) but not normal tissue. Further specificity can be obtained for specific histologic types of tumor through the construction of engineered viruses with virus essential genes linked to tumor-type-specific enhancer/promoter elements. These technical hurdles, while complex, have begun to be resolved with the molecular understanding of both cancer cells and the many genes of HSV-1.

Targeted viral entry

The simplest way to achieve tumor cell specificity involves altering the way the virus recognizes cells (i.e. receptor-mediated binding and fusion). This involves retargeting the virus so that it binds only to tumor cell-specific receptors. This approach has not currently been undertaken with replication-competent HSV. However, recent years have seen much advancement in the understanding of the mechanism of HSV-1 entry into cells, which can be exploited to target HSV-1 entry to tumor cells [6,7].

Targeting proliferating cells

One method of obtaining replication-conditional HSV-1 is to create viral mutants that have deletions that can be complemented by certain cells. Certain viral genes encode proteins that have cellular homologs and therefore could be complemented by the tumor cell. The use of HSV-1 as an anti-glioma agent provides a case study for this type of approach. Glioma cells grow in an environment surrounded by quiescent neurons. HSV-1 selectivity for gliomas is achieved by exploiting the differences between the intracellular milieu of dividing glioma cells and that of quiescent neurons. HSV-1 encodes multiple genes involved in viral DNA replication as well as nucleotide precursor production. These genes include viral thymidine kinase and the large subunit of ribonucleotide reductase [1]. Such genes may be necessary for wild-type virus to efficiently replicate in cells where deoxynucleotide pools are low

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(e.g. quiescent neurons). However, tumor cells may have large pools of deoxynucleotides that the virus can scavenge to replicate its DNA. Thus, deletion of viral genes involved in DNA synthesis has provided a rational basis to create genetically engineered HSV-1 as an anticancer agent.

Based on this rationale, the HSV-1 viral mutant *dl*sptk containing a deletion in the gene encoding thymidine kinase was initially shown to have efficacy against experimental gliomas [3]. However, deletion of viral thymidine kinase has the drawback of removing the ability to treat adverse effects of infection, should they occur in patients, with acyclovir. Using an analogous strategy, an HSV-1 viral mutant hrR3 containing the *Escherichia coli lacZ* gene inserted within the gene encoding the large subunit of ribonucleotide reductase (U_L39) was utilized for experimental glioma animal models [8]. This virus has the advantage of being ten-fold more sensitive to both acyclovir and ganciclovir. Furthermore, the expression of the *lacZ* gene allows detection of the virus within histologic sections of the tumor. In a similar approach, HSV-1 with a deletion of the U_L2 gene encoding uracil DNA glycosylase was shown to have efficacy against tumor xenografts as well as ganciclovir hypersensitivity [9]. These initial studies have spawned much interest in the use of replication-conditional HSV-1 as antiglioma agents and more broadly as antitumor agents for a wide variety of tumors. However, many normal tissues also have high mitotic indices, and such viruses may not discriminate between rapidly proliferating normal and cancer cells.

Creating a non-neurovirulent replication-competent HSV-1

The discovery of an HSV-1 gene associated with neurovirulence has provided further impetus for the use of replication-conditional HSV-1 for cancer. Deletion of the $\gamma_1 34.5$ gene, present in two copies in the long repeat regions, reduced neurovirulence by greater than five orders of magnitudes upon direct intracranial inoculation in mice [10]. In response to viral infections, cells activate RNA-dependent protein kinase (PKR), which phosphorylates the α subunit of translation initiation factor 1 (eIF-2α). Phosphorylated eIF-2α blocks the translation of mRNA, and thus provides an innate host defense mechanism against viral infection. HSV-1 has evolved a rather curious mechanism to deal with PKR activation. The product of the $\gamma_1 34.5$ viral gene binds to protein phosphatase-1, resulting in dephosphorylation of eIF-2α, and precluding host protein synthesis shut-off [11]. Given that HSV-1 deleted in $\gamma_1 34.5$ (R3616) is severely attenuated for neurovirulence, this virus is an ideal candidate for tumor therapy within the central nervous system (CNS). Subsequently, R3616 has been shown to be safe and also effective in intracranial glioma and xenograft models [12,13].

However, viruses deleted for $\gamma_1 34.5$ replicate sluggishly and are not genetically stable upon serial passage in cell culture, since second-site mutations in this virus that overcome the protein synthesis shut-off characteristic of $\gamma_1 34.5$ viral mutants have been isolated [14]. One compensatory second-site mutation in HSV-1 lacking $\gamma_1 34.5$ is located in the $U_S 11–12$ region [15]. The $U_S 11$ gene is normally expressed as a late gene with γ2 kinetics, whereas the $U_S 12$ gene (ICP47) is expressed with immediate early (α) kinetics [1]. Viral mutants that link the $U_S 11$ gene with the $U_S 12$ promoter express $U_S 11$ with immediate early kinetics rather than γ2 kinetics. When $U_S 11$ is expressed earlier, it is effective in compensating for the deletion in $\gamma_1 34.5$, in that eIF-2α is not phosphorylated and protein translation does not cease [16,17].

There are at least two phenotypes associated with $\gamma_1 34.5$-deleted HSV-1, eIF-2α phosphorylation and decreased neurovirulence. If these phenotypes are the result of distinct functions of $\gamma_1 34.5$, then it may be possible to prevent eIF-2α phosphorylation by expressing $U_S 11$ with immediate early kinetics in a $\gamma_1 34.5$-null virus while still retaining decreased neurovirulence. Predictably, such a virus would replicate more efficiently within tumor cells (since protein translation is not blocked) and would result in enhanced tumor regression. Recent evidence supports this hypothesis. Viruses containing deletions in $\gamma_1 34.5$ and expressing $U_S 11$ from the $U_S 12$ promoter replicated nearly as efficiently as wild-type virus in tumor cell lines that fail to support appreciable growth of the $\gamma_1 34.5$-null virus. The extent of replication correlated with enhanced tumor xenograft regression [18,19]. Importantly, this virus still retained the non-neurovirulent phenotype in mice associated with the $\gamma_1 34.5$-null virus, and it may serve as a more successful oncolytic agent. However, serial passage of $\gamma_1 34.5$-null mutant virus in human cells yielded additional second-site compensatory mutants that were able to synthesize

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viral proteins and exhibited increased neurovirulence [20]. It is likely that serial passage of $\gamma_1 34.5$-null mutants in the central nervous system would also select for heightened neurovirulence.

Transcriptional targeting of tissue-specific cells

As mentioned above, some normal tissues have mitotic indices that rival those of tumors. Thus, another approach to target tissue-specific tumor cells has been recently explored. Transcriptional differences exist between different organs as well as between tumor and normal cells of the same organ. Identifying specific cell gene products transcriptionally upregulated in cell types allows for the generation of engineered HSV-1 mutants that contain such promoter/enhancer elements upstream of essential viral genes. Such a strategy restricts viral replication to cells that contain transcription factors to drive the expression of the chimeric cellular promoter/viral essential gene.

Studies have shown that such an approach is feasible with the *α4* gene of HSV-1. The *α4* gene exists in two copies in the HSV-1 unique short repeat regions, is essential in cell culture, and is expressed immediately after infection [1]. Infected cell protein number 4 (ICP4), the product of the *α4* gene, is a major viral regulatory protein and transactivator. The HSV-1 viral mutant d120 contains deletions in both copies of ICP4, but results in apoptosis upon infection of cells [21,22]. To target albumin-expressing cells, virus G92A was created by placing the *α4* gene under the transcriptional control of the albumin enhancer and promoter elements and inserting it into d120 [23]. While wild-type HSV-1 replicated efficiently irrespective of albumin expression, virus G92A replicated 3 log more efficiently in albumin-expressing cell lines than in non-albumin-expressing cell lines. However, G92A replicated more slowly than wild-type HSV-1, which in part is due to the delayed accumulation of ICP4 in G92A-infected cells as compared to wild-type infected cells. Importantly, G92A was also shown to selectively inhibit tumor xenograft growth of albumin-expressing cells (i.e. hepatomas) in animal models compared to non-albumin-expressing tumor xenografts (PC-3 or HeLa) [24]. There was no apparent HSV-1 toxicity in mice intrahepatically injected with G92A. The problem with this approach is that albumin is transcriptionally expressed in the normal adult liver, indicating that normal liver cells may support the replication of G92A by expression of ICP4 from the albumin enhancer/promoter elements, with resultant destruction of normal liver cells. A similar approach for targeting soft tissue and bone tumors through the use of the human calponin promoter driving the expression of the *α4* gene in a d120-based virus has been reported [25]. Calponin mRNA and protein are aberrantly expressed in a variety of human soft tissue and bone tumors; these tumor cells could support the replication of such a virus, resulting in tumor-type-specific oncolysis.

With the advent of gene microarrays, it is feasible to quickly screen and identify genes that are transcriptionally upregulated in tumor cells compared to normal tissue. By using the above-mentioned technique, tumor-type-specific HSV-1 could be tailored for individual tumor histologies. However, such a strategy requires that multiple viruses be created and driven by tissue/tumor-specific promoters. Also, since tumors consist of a heterogeneous population of cells, specific promoter elements may not be active in all the cancer cells. Finally, leaky enhancer/promoter elements may allow replication in non-targeted cells.

Replication-competent HSV-1 in clinical cancer trials

With a wealth of preclinical studies having been done with replication-competent, genetically engineered HSV-1 over the last decade, such constructs have entered clinical trials. Safety is of utmost concern in using replication-competent viruses, since wild-type HSV has been the cause of disease since ancient times, particularly life-threatening encephalitis. To date, at least three phase I clinical trials have been completed or are underway using replication-competent, genetically engineered HSV-1 for tumor therapy. Three different attenuated HSV-1 viruses were used in each of these studies and are identified as 1716, G207, and NV1020 (R7020). Both 1716 and G207 have completed phase I trials in patients with recurrent gliomas in the UK and the USA, respectively. NV1020 is currently undergoing phase I trials in patients with liver metastases from adenocarcinoma of the colon.

1716 and G207

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor.

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Characteristics of this tumor make it an ideal candidate for experimental therapy. First, the median survival from diagnosis is 1 year with such conventional treatments as surgery and external beam radiotherapy. Second, treatment failure is local, in that 90% of patients have tumor recurrence within 2 cm of the initial lesion, and metastases (systemic spread) are rare. Given the rapid mortality and local therapy failure, GBM was the first tumor to be tested with genetically engineered HSV-1. HSV-1 deleted in both copies of $\gamma_1 34.5$ is significantly neuroattenuated and resulted in the regression of experimental brain tumors in animal models [10,12,13,26]. This construct with the addition of a mutation in the ribonucleotide reductase gene provides the construct for G207, which was the basis for a phase I clinical trial in the USA [27]. G207 (strain F derived) is deleted in both copies of $\gamma_1 34.5$ and has a *lacZ* insertion in the $U_L 39$ gene that inactivates the large subunit of ribonucleotide reductase (ICP6). The $\gamma_1 34.5$ deletion renders the virus non-neurovirulent (identical to 1716), and inactivation of ICP6 targets the virus to replicate in proliferating cells. For safety reasons, it may be beneficial to create attenuated viruses that are multimutated to reduce the probability of second-site mutations that can restore functions of deleted genes, as exemplified by altered expression of $U_S 11$ complementing the protein synthesis shut-off phenotype of $\gamma_1 34.5$. G207 has been repeatedly found to have oncolytic activity against a variety of tumor histologies, and its safety has been characterized in *Aotus nancymae* (New World owl monkeys) by intracerebral inoculation at doses up to 10^9 PFU [27–30]. Twenty-one patients with malignant glioma were enrolled in a dose-escalating study with G207, starting at 10^6 PFU and rising to 3×10^9 PFU [31]. No patients developed HSV encephalitis or adverse toxicity directly ascribed to G207 following intratumoral inoculation of G207, even at the highest dose. An HSV-1-seronegative patient treated at the highest dose did seroconvert. Tissue samples from two patients were positive for HSV-1 and *lacZ* by PCR, indicating the presence of G207.

The genetically engineered HSV-11716 (strain 17 derived) is deleted in both copies of $\gamma_1 34.5$ [32]. A phase I clinical trial has been completed in Glasgow, assessing the safety of 1716 upon intratumoral inoculation in nine patients with relapsed GBM [33]. Groups of three patients received 10^3, 10^4 or 10^5 PFU by stereotactic intratumoral injection. No evidence of encephalitis, reactivation of HSV-1 or adverse clinical outcomes was seen in this clinical trial. Tissue samples following viral inoculation were available from three of the patients who underwent subsequent tumor resection. The earliest time was 3.5 weeks postinoculation. Unfortunately, immunohistochemistry and PCR analyses of the DNA from these tissues failed to reveal the presence of either HSV-1 or 1716. Both of these were successful phase I clinical trials, in that they established the safety of stereotactic intracranial inoculation of replication-competent, genetically engineered HSV-1 for experimental therapy of malignant glioma. This is no small feat, recognizing the specific potential for the development of encephalitis associated with this treatment paradigm. At the highest doses employed in each trial, no adverse effects were directly attributable to the viral therapy. Notably, the inoculum was 4 log greater in the US study. Both trials used a $\gamma_1 34.5$-null virus, with the difference between the viruses being whether ICP6, the large subunit of ribonucleotide reductase, was functional (1716) or inactivated (G207). Each strategy has its own advantages and disadvantages that have to be weighed in terms of antitumor efficacy and patient safety. While deletion of $\gamma_1 34.5$ creates a highly attenuated virus in terms of neurovirulence, such viruses can eventually evolve second-site mutations to overcome the protein synthesis shut-off phenotype. Given that the molecular basis for attenuated neurovirulence of $\gamma_1 34.5$-deleted HSV-1 is unresolved, certain safety precautions should be built into the virus. This was in part the basis for inactivation of ICP6 in G207. ICP6 inactivation also has the benefit of being hypersensitive to ganciclovir in cases of adverse sequelae to HSV-1 inoculation.

However, $\gamma_1 34.5$-deleted viruses also do not replicate to very high titers in experimental tumor models. Inactivation of ICP6 would result only in a less replication-competent virus. Since tumor volume is the net effect of tumor cell division and death, effective clinical responses would require a virus whose replication outstrips tumor growth. Deletion of viral thymidine kinase or ribonucleotide reductase creates a replication-conditional virus that targets proliferating cells such that it can scavenge nucleotide precursors from the cell. G207 may effectively replicate in the fraction of cancer cells within a tumor that are actively dividing. However, a significant proportion of

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cells within a tumor may not divide at a sufficient rate to complement the virus in *trans* with nucleotide precursors, leading to abortive infection. This may explain in part why the Glasgow trial dose escalated to 10^5 PFU with 1716 compared to 3×10^9 PFU with G207. Many questions have arisen as a result of these trials. It appears that toxic doses of 1716 or G207 were not attained, implying that larger viral inocula could be tolerated. While a greater PFU inoculum of 1716 may be given in a follow-up Glasgow trial, it is unlikely that viral stocks made by good manufacturing practices will allow for delivery of more than 3×10^9 PFU in future G207 clinical trials. Owing to the location of these tumors, it is also difficult to assess whether viral replication occurred within the tumor. Tissue was available in these studies following viral inoculation only when re-resections were performed or at autopsy. These phase I trials, designed to established safety, will lead to more in-depth trials that will begin to address these questions as well as the most important question of clinical efficacy.

NV1020

While the above phase I clinical trials were completed, a different attenuated, replication-competent genetically engineered HSV-1 mutant has entered clinical trials for liver metastases from colon adenocarcinoma (http://www.clinicaltrials.gov). NV1020 is a clonal derivative of HSV-1 R7020. Genetically engineered HSV-1 R7020 is an intertypic virus, initially constructed for immunization against both HSV-1 and HSV-2 [34]. It contains deletions of the internal inverted repeat (one copy of *α0*, *α4*, *$\gamma_1$34.5*, *ORF O*, and *ORF P*) as well as *U_L23* (thymidine kinase), the *U_L24* promoter, and *U_L56*. Into the virus carrying these deletions were inserted the *U_L23* encoding viral thymidine kinase under the control of an *α* promoter as well as the genes encoding the HSV-2 glycoproteins G, J, D, and I. R7020 was extensively studied for genetic stability and safety in rodents, rabbits, and primates [34,35]. Given that vaccines are created with the utmost regard to safety for healthy patients, this virus appears to satisfy the safety criteria for a potential oncolytic agent for patients with cancer. While this virus retains one copy of the *$\gamma_1$34.5* gene, it is attenuated for neuroinvasiveness, and its attenuation appears to be related to the absence of an internal inverted repeat. Thus, non-CNS inoculation of the virus may be safe. Initial studies with R7020 revealed that it grew more robustly in tumor xenografts than R3616 [36]. In experimental tumor models, intratumoral injection of equivalent PFU of R7020 or R3616 resulted in the recovery of 25-fold more R7020 than R3616. This result led to studies demonstrating the antitumor efficacy of R7020 for multiple non-CNS tumors (i.e. head and neck squamous cell carcinoma, prostate adenocarcinoma, pancreatic carcinoma, and metastatic hepatic cancer) [37–39]. Additionally, the non-neuroinvasive phenotype of R7020 following intratumoral replication was proven in susceptible murine models. Whereas wild-type HSV-1 injection of flank tumors resulted in both tumor regression and also death of the mouse, R7020 injection resulted in tumor regression without death of the mouse [37]. Given that NV1020 (R7020) is multimutated, non-neuroinvasive, and replicates more robustly than R3616 or G207, it provides a second genetically engineered HSV-1 antitumor agent for non-CNS tumors. NV1020 is currently in phase I clinical trials for colon adenocarcinoma metastases to the liver. Patients will receive a single intrahepatic arterial injection of R7020 for their liver metastases, with dose escalation to evaluate safety.

Multimodality therapy with replication-competent HSV-1 for cancer therapy

While proof has been demonstrated in principle for the use of genetically engineered replication-competent HSV-1 for oncolysis in experimental tumor models, strategies have evolved to further improve the therapeutic ratio of these viruses. One involves the insertion of therapeutic genes into the virus genome, and the other involves combining viral therapy with standard cancer therapies (i.e. chemotherapy or radiotherapy). Such combinations result in the destruction of tumor cells by a variety of mechanisms, as well as interactive effects of the therapies. Also, lower doses of each therapy in combination may enhance tumor control while minimizing normal tissue toxicity caused by the therapies. However, such approaches, on the surface, appear counter-intuitive, in that the adjuvant therapy may lead to destruction of infected cells prior to the completion of the viral lytic cycle, and thereby limit the efficacy of replication-competent viruses. Thus, it becomes important to determine the impact of the additional

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therapy on the ability of the virus to replicate within the tumor.

Foreign gene insertion within replication-competent HSV-1

Immune modulation

Cancer immunotherapy, like gene therapy, has been the focus of intense clinical investigations. Many cancer patients are immunosuppressed, and tumor cells have been shown to secrete immunosuppressive molecules (i.e. TGF-β and prostaglandins); also, tumor-specific antigens have been identified for certain tumors. However, systemic administration of immune-stimulating cytokines is accompanied by dose-limiting toxicity. Viral vectors, on the other hand, offer the opportunity to spatially restrict cytokine expression directly within the inoculated tumor bed. Viral replication, local overexpression of cytokines and tumor cell debris following viral lysis may all combine to result in a potent antitumor immune response. This concept was shown in principle by the insertion of interleukin-4 (IL-4) into γ_1*34.5*-deleted HSV-1 [40]. Cells infected with IL-4-encoding virus showed a 1300-fold increase in production of IL-4. Intracranial inoculation of this virus in a syngeneic murine glioma model also resulted in an increased accumulation of macrophages and CD8- and CD4-positive T-cells, along with enhanced survival.

IL-12 has also been inserted into γ_1*34.5*-deleted HSV-1 virus, M002 [41]. Certain known properties of IL-12 may make it a better agent for immunotherapy. IL-12 is a heterodimer consisting of 35- and 40-kDa subunits. The actions of IL-12 include proliferation of cytotoxic T-cells and natural killer cells, cell-mediated immunity (by promoting Th1 development), and antiangiogenesis. Since IL-12 is a heterodimer, M002 was constructed with a γ_1*34.5*-deleted virus and insertion of IL-12 in a single expression cassette, with the p40 and p35 subunits separated by an internal ribosomal entry site. Infection of cells with M002 resulted in the expression of physiologic levels of IL-12 [41]. Moreover, in a murine syngeneic model for neuroblastoma, M002 resulted in prolonged survival and revealed an abundance of CD4+ and CD8+ T-cells and macrophages compared to the parental γ_1*34.5*-deleted HSV-1.

In employing immunomodulation in conjunction with replication-competent HSV-1, the effects of HSV-1 infection on immune evasion should be dealt with. ICP47, the product of the U_S*12* gene, has previously been shown to block MHC class I expression of antigenic peptides [42]. ICP47 binds to the transporter associated with antigen processing (TAP) and prevents peptide translocation in the endoplasmic reticulum and loading of MHC class I molecules. In strategies employing replication-competent HSV-1 with immunomodulatory cytokines for cancers, deletion of the U_S*12* gene may prove beneficial. Deletion of the U_S*12* gene is also reported to decrease neurovirulence (i.e. enhance survival) in mice infected though the cornea, a finding which may improve viral safety [43]. Using the viruses in current clinical trials as a backbone, U_S*12* has been deleted from both G207 and NV1020 [19,44]. As mentioned above, deletion of the U_S*12* gene and placement of the U_S*12* α promoter in juxtaposition with the U_S*11* gene has been used to abrogate phosphorylation of eIF-2α associated with γ_1*34.5*-deleted virus (G207), resulting in more robust viral replication [19]. As a consequence of the deletion of U_S*12*, MHC class I surface expression is not blocked in infected cells [19]. A similar approach has been studied with NV1020. The viruses NV1034 and NV1042 do not produce ICP47, due to the insertion of the *Escherichia coli lacZ* gene within the U_S*12* gene, and encode either GM-CSF or IL-12, respectively [44]. In studies using a syngeneic murine squamous cell carcinoma model, pre-established tumors injected with NV1042 (mIL-12 expressing) had enhanced regression compared to the parental virus, which does not encode mIL-12. Importantly, these studies also demonstrated memory immunity to the tumor from NV1042 treatment. Thus, mice with established flank tumors treated with NV1042, and rechallenged with the same tumor cells in the contralateral flank, developed significantly fewer tumors.

Prodrug-converting enzymes

This paradigm involves delivery of a gene encoding a prodrug-converting enzyme (mammalian or non-mammalian) to tumor cells, which is then followed by systemic administration of a prodrug. Given that the prodrug is relatively innocuous, it circulates within the bloodstream and is converted to a toxic metabolite in cells expressing the prodrug-converting enzyme. This approach has also been termed 'suicide gene therapy'. Certain drugs can diffuse out of the cell that encodes the

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prodrug-converting enzyme. Thus, neighboring cells that have not been transduced with the prodrug-converting enzyme can also be killed by the soluble toxic metabolite (bystander effect), greatly increasing the utility of this treatment paradigm.

HSV-1 encodes one such enzyme, thymidine kinase (HSV-1 tk). This enzyme has been extensively studied in combination with ganciclovir or acyclovir. HSV-1 tk has been delivered by numerous transfection agents and has laboratory efficacy for tumor cell destruction. Almost all current replication-competent HSV-1 viruses that are being studied encode HSV-1 tk. This enzyme provides a 'safety net', in that administration of ganciclovir or acyclovir can inhibit viral replication and enable cessation of therapy with replication-competent HSV-1. Two prodrug-converting enzymes, cytochrome P450 2B1 and cytosine deaminase, have also been inserted in replication-competent HSV-1, with the goal of improving tumor cell kill, while not impairing the ability of the virus to replicate [45,46].

Cytochrome P450 2B1 converts cyclophosphamide and ifosfamide to toxic metabolites. In converting cyclophosphamide, cytochrome P450 2B1 produces phosphoramide mustard (a DNA alkylator) and acrolein (a protein alkylator). Normally, cytochrome P450 2B1 is expressed in hepatocytes but not in tumor cells, such that cyclophosphamide is activated in the liver, resulting in systemic distribution of these toxic products to normal tissues with high proliferative indices, gastrointestinal mucosa and hematopoietic cells. By transducing tumor cells with cytochrome P450 2B1, elevated intratumoral concentrations of cyclophosphamide metabolites may be achieved, resulting in enhanced tumor cell kill with decreased normal tissue toxicity. The replication-competent HSV-1 rRp450 was constructed by insertion of the rat cytochrome *P450 2B1* gene into the U_L39 gene locus, resulting in loss of expression of the viral large subunit of ribonucleotide reductase (ICP6) [45].

Cytosine deaminase (CD) is expressed by bacteria and fungi, but not in mammalian cells, and deaminates cytosine to uracil. CD can therefore convert non-toxic 5-fluorocytosine (5-FC) to the common anticancer agent 5-fluorouracil (5-FU). 5-FU is metabolized to a deoxynucleotide monophosphate (5-dUMP) and poisons thymidylate synthetase, which inhibits DNA synthesis. 5-FUTP produced from phosphorylation of 5-FU can also get incorporated into RNA and disrupt mRNA and rRNA function. Though 5-FU is an effective cytotoxic agent with systemic toxicities, the therapeutic window of this drug could be enhanced by confining 5-FU production to the tumor bed through the CD/5-FC combination. 5-FU is also freely diffusible, such that surrounding non-CD-transduced cells can be exposed to 5-FU. The replication-competent HSV-1 HSV1yCD was created by insertion of the yeast *CD* gene into the U_L39 gene, thereby inactivating ICP6 [46].

By virtue of the inactivation of ICP6 in rRp450 and HSV1yCD, both viruses have a predilection to replicate in tumor cells compared to normal cells [8]. Treatment with either of these prodrug-converting enzyme-containing viruses enhanced tumor cell cytotoxicity and tumor xenograft regression upon administration of the prodrug [45–47]. Importantly, the toxic metabolites of cyclophosphamide or 5-FC do not significantly attenuate the ability of the virus to replicate, whereas treatment with ganciclovir drastically reduced viral replication [46,47].

Replication-competent HSV-1 combination with standard cancer therapies

Many current cancer therapeutic regimens involve the use of combinations of surgery, radiotherapy, and chemotherapy. The application of replication-competent genetically engineered HSV-1 in combination with either radiotherapy or chemotherapy has also been explored. As stated above, the only proven beneficial therapy for GBM is the use of radiotherapy in concert with surgery. Given that radiotherapy is routinely employed for GBM as well as in over 50% of all cancer patients, and considering its ability to be specifically targeted to the tumor bed, it may prove to be a useful complement to intratumoral HSV-1 inoculation. The combination of ionizing radiation with either R3616 or R7020 has been shown to result in enhanced tumor regression and survival in experimental tumor models for gliomas or non-CNS histologic tumor xenografts [36,37,48]. As for the mechanism of enhanced tumor regression with this combined therapy, studies indicate that ionizing radiation may result in enhanced viral replication and distribution of replication-competent genetically engineered HSV-1 within experimental tumor xenografts [36,48].

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A variety of chemotherapeutic agents have also been tested in combination with replication-competent HSV. The chemotherapeutic agent cisplatin has been utilized in combination with G207 for human squamous cell carcinomas [49]. Cisplatin did not adversely affect viral growth, and in certain tumor cell xenografts, cisplatin combined with G207 resulted in supra-additive effects on xenograft regression. Mitomycin C in combination with 1716 for non-small cell lung cancer xenografts had additive effects [50]. Finally, fluorodeoxyuridine (FUdR) has been shown to enhance replication and cytotoxicity of G207 in colorectal adenocarcinoma cell lines [51]. The mechanism of action of FUdR appears to aid G207 replication by targeting the activity of cellular ribonucleotide reductase (RR). As stated before, G207 is deleted in $\gamma_1 34.5$ and lacks functional ICP6 (large subunit of ribonucleotide reductase). One mechanism of action of FUdR is inactivation of cellular thymidylate synthetase, resulting in depletion of dTTP and imbalance of intracellular deoxynucleotide pools. A consequence of FUdR is the upregulation of cellular RR activity. Thus, the upregulation of cellular RR upon treatment with FUdR can aid in complementing the ICP6 defect of G207.

With the apparent safety of 1716 and G207 demonstrated in the recently completed phase I clinical trials, the addition of standard radiotherapy or chemotherapeutic agents in future clinical trials may be warranted. Such combinations of standard cancer therapy with replication-conditional virus also has a precedent in knowledge gained with the ONXY-015 adenovirus. In a phase II clinical trial of patients with recurrent head and neck cancers, the virus proved especially beneficial in combination with chemotherapy (cisplatin and 5-fluorouracil) [52]. While the mode of action of enhanced tumor cell eradication in combination with replication-competent HSV-1 is speculative (except, perhaps, for FUdR), these therapies are standard cancer treatments, and may allow for enhanced therapeutic ratios, resulting in improved clinical efficacy.

REPLICATION-DEFECTIVE HSV-1

Given the relatively large genomic size of HSV-1, a benefit of utilizing replication-defective HSV-1 over other viral transfection systems is the ability to insert large and/or multiple foreign genes into these vectors. Inherent neurotropism also provides a means to deliver genes to neurons or other postmitotic cells that are difficult to transduce by other viral vectors. In addition, HSV-1 viruses have a wide-range cell tropism, and the genome remains extrachromosomal, thereby minimizing the risk of genetic changes in the infected cell by insertional mutagenesis. Replication-defective HSV-1 mutants were initially categorized as either helper virus dependent or independent. The helper virus-dependent viruses are also termed amplicons, and minimally consist of packaging sequences (the α terminal repeats) and an origin of viral DNA replication. Amplicons will not be discussed in this review, and the reader is referred to recent reviews on this topic [53,54]. Helper virus-independent viruses (referred to here as replication-defective HSV-1) have deletions in one or more essential genes and can be grown in cell lines that express the essential gene to provide the gene product in *trans*. Recombination to wild-type virus can be minimized by creating cell lines with minimal essential gene coding fragments that have no homology to the deleted virus genome.

Replication-defective essential gene-deleted vectors

To propagate HSV-1 mutants with deletions of essential genes, cell lines are created to express the deleted viral gene and provide it in *trans* to the mutated virus, so that replication can proceed with resultant mutant progeny. The advantages of this system are two-fold. There is no risk of contamination with helper viruses, and high viral titers can be produced easily. Up to 40 kb of foreign DNA could be inserted into such viruses, depending on the deletion of non-essential genes as well. Two of the immediate early genes of HSV-1 are essential, $\alpha 4$ and $\alpha 27$. Deletion of either of these genes would produce a potential replication-defective HSV-1 to be used as a vector for gene delivery. However, it was soon realized that mutant viruses harboring deletions in either $\alpha 4$ or $\alpha 27$ were cytotoxic and induced apoptosis of the infected cell, thereby precluding their use as stable gene transfer vectors [21,55–57]. While the absence of ICP4 or ICP27 production inhibits viral replication, certain viral proteins (mainly the remaining immediate early) are still produced by infection with these mutants. Two strategies could be employed to circumvent the problem with a single deletion virus. The first

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would be to attenuate the expression of the remaining viral genes expressed in a single deletion virus (assuming that expression of the remaining α genes is toxic to the cell), and the second would involve blocking the cytotoxicity of a single deletion virus by insertion of a protective gene into the mutant virus.

A virus deleted for *α4* (d120) continues to produce the remaining α proteins (ICP0, ICP22, ICP27, and ICP47) as well as a few other genes [58]. The toxicity due to d120 could be in part due to the expression of these genes. Since α gene transcription is transactivated by the virion tegument protein VP16 (αTIF), an *α4*-null virus was created with a mutation in VP16 [59]. This virus was less cytotoxic, with increased cell survival compared to an *α4*-null virus. A more direct approach to address the cytotoxicity of the *α4*-null virus would be the systemic deletion of the remaining α genes. These results showed that a virus deleted in *α4*, *α22* and *α27*, or *α0*, *α4* and *α27*, had increased cell survival compared to a virus deleted in *α4* alone [58,60]. And when all five of the immediate early genes were deleted, the viral vector exhibited almost no cytotoxicity as measured by cell survival, providing an innocuous gene transfer vector [60].

The second approach to attenuating the cytotoxicity of *α4*-null viruses would be to protect the infected cell from its apoptotic fate. The *α4*-null viral mutant d120 has previously been shown to induce apoptosis [21]. Apoptosis induced by d120 can be blocked by the overexpression of two proteins, Bcl-2 or U_S3 (HSV-1-encoded viral kinase). d120 infection of a cell line that overexpresses Bcl-2 did not result in apoptosis [22]. Also, baculoviral delivery of the U_S3 blocked d120-induced mitochondrial cytochrome *c* release and procaspase 3 cleavage [61]. Therefore, a d120-based vector encoding either of these genes may allow for gene transfer without induction of apoptosis. However, there is leaky late gene expression from d120 that is likely to be toxic to the cell, which necessitates the deletion of other viral α genes to create a non-toxic vector.

The goal of such vector design is the ability to infect cells and drive long-term gene expression of a therapeutic gene. While a virus deleted in all five α genes is not cytotoxic, CMV IE promoter-driven foreign gene expression delivered with this virus is also severely inhibited [60]. The CMV promoter may not be suitable for such a mutant virus, since it has been previously shown to be transcriptionally repressed in HSV-1 mutants that did not express immediate early proteins [62]. To circumvent this problem, studies have been done with an HSV-1 virus deleted in *α4*, *α22*, and *α27* [63]. Such a virus was able to express the reporter *lacZ* gene driven from either the CMV IE promoter or HSV-1 ICP0 promoter for up to 21 days after infection of neurons in cell culture. The HSV-1 virus deleted in *α4*, *α22* and *α27* has also been shown to be effective in delivering neuroprotective genes in an animal model. In a rat model for Parkinson's disease, in which death of neurons in the substantia nigra is induced with the chemical 6-hydroxydopamine, a replication-defective HSV-1 deleted in *α4*, *α22* and *α27* encoding *Bcl-2* driven by the CMV IE promoter was able to significantly enhance neuronal survival compared to the virus that does not encode *Bcl-2* [64]. While encouraging, this animal model is problematic, since it induces fairly rapid death of substantial nigra neurons, and the virus is given 1 week prior to the neuronal insult. It would be beneficial to determine the length of time *Bcl-2* can be expressed after viral inoculation. Studies have begun to address the issue of how different promoters temporally express transgenes in defective viruses. In an ICP4-null virus, nerve growth factor (NGF) was placed under the control of either the CMV IE promoter or the latency active promoter 2 (LAP2). The CMV IE promoter maximally expressed NGF 3 days post-infection, whereas the LAP2 promoter had greater expression of NGF at 14 days post-infection of primary dorsal root ganglia cultures [65]. This result is intriguing in that it suggests that the best way to prevent neuronal insult in the short and long term would be to employ a combination of viruses (or a single virus with two expression cassettes) with both an immediately active promoter and a promoter that would transcribe at a later time. Thus, for immediate protection, a CMV or HSV-1 IE promoter would quickly transcribe the protective gene (days 1–7 post neuronal insult, for example), after which transcription from the LAP promoter would protect during the following weeks.

Another advantage of replication-defective HSV mutants is their ability to insert large genes or multiple genes. A replication-defective HSV-1 has been deleted for 11.6 kb. It is deleted in the α genes *α4*, *α22*, *α27*, and *α47*, ensuring decreased vector toxicity, along with deletions in the genes U_L24, U_L41, U_L44, U_S10, and U_S11. One way to enlarge the capacity of replication-defective HSV-1

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is by simultaneous deletion of non-essential viral genes. In this multimutated HSV-1, genes encoding IL-2, GM-CSF, B7.1, and LacZ or IFN-γ were inserted, for a total 12-kb insertion [66]. The four genes were inserted as separate transcriptional units and were simultaneously expressed in cell culture upon infection of primary melanoma cells for up to 1 week. Also, the rather large, 14-kb *dystrophin* gene was placed into replication-defective HSV-1, for potential treatment of muscular dystrophy [67]. The virus was able to express dystrophin upon infection of muscle cells in culture, and in a dystrophin-deficient mouse model (MDX), the virus was able to express dystrophin in muscle cells at the site of viral inoculation. Insertion of such a large single gene would be a rather difficult task for other viral delivery systems.

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